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The nature of DNA breakage by 4′-[(9-acridinyl)amino]methane-sulphon-*m*-anisidide

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DNA fragment *mAMSA* *5′-Terminus–protein association* *(T4 polynucleotide kinase)* *Topoisomerase* *(Mouse mastocytoma PY 815 cells)*

1. INTRODUCTION

Although recent studies have shown that the anticancer drug 4′-[(9-acridinyl)amino]methane-sulphon-*m*-anisidide (mAMSA) induces double-strand breaks in the DNA of treated cells [1,2] the exact sites or nature of these breaks in DNA are unknown. Zwelling et al. [1] have concluded that the DNA isolated from mAMSA-treated cells is associated with protein because it binds to polyvinyl chloride filters during the alkaline-elution procedure [3] and they have suggested that intercalation of mAMSA into cellular DNA may break the DNA by inducing topoisomerase action leading to the association of DNA fragments with topoisomerase. A similar suggestion has been made regarding other intercalating drugs [4]. In view of these suggestions it is of considerable interest to determine whether the termini of DNA fragments produced following mAMSA action are in fact associated with proteins in the manner expected if topoisomerases sever the DNA. To examine this question we have used phage T4 polynucleotide kinase to assess the availability of 5′-termini on DNA fragments produced after treating PY815 mouse mastocytoma cells in culture with mAMSA. Our results indicate that the DNA 5′-termini are not available for phosphorylation consistent with the view that they may be linked to protein.

2. MATERIALS AND METHODS

2.1. *Materials*

T4 polynucleotide kinase was a product of New England Biolabs. Pancreatic deoxyribonuclease I was from Calbiochem. Snake venom phosphodiesterase, ribonuclease T1 and pancreatic ribonuclease were from Worthington Biochemical Corp. Calf intestinal alkaline phosphatase was from Sigma Corp., Phage λ DNA was obtained from Miles Laboratories and Proteinase K from Boehringer. [γ-^{32}P]ATP (spec. act. 5700 Ci/mmol) was from Amersham, U.K.

2.2. *Cell culture and mAMSA treatment*

Mouse mastocytoma PY815 cells were grown and treated with 4 μM mAMSA for 30 min as previously described [2].

2.3. *Preparation of PY815 cell DNA*

Log phase PY815 cells (1×10^7 cells) both untreated and treated with mAMSA were centrifuged at 500 × *g* and washed twice with ice-cold physiological buffered saline. The final pellet of cells was resuspended in the same buffer to a density of 2×10^6 cells/ml and an equal volume of lysis solution containing 20 mM Tris–HCl (pH 7.6), 20 mM Na EDTA, 1% lithium dodecylsulphate, 20 mM NaCl and 100 μg/ml proteinase K was added. The lysed cells were rocked gently for 18 h at 20°C then an equal volume of 20 mM Tris–HCl-saturated phenol was added and the DNA was extracted by gently mixing the solutions for 1 h. After centrifugation at 8000 × *g* for 15 min the aqueous phase was removed with a very wide-bore pipette taking care to avoid the interface and nucleic acids were precipitated by adding 2 vol. of 90% ethanol at 0°C. The precipitates were washed twice with 70% ethanol containing 0.1 M Na^+ acetate and resuspended in 10 mM Tris–HCl, 1 mM

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EDTA (pH 7.6). Pancreatic ribonuclease and ribonuclease T1 (each 5 μg/ml and preheated to destroy deoxyribonucleases) were then added at 25°C for 1 h to hydrolyse any associated RNA. The DNA was again precipitated with ethanol, washed with 70% ethanol, resuspended in 1 mM Tris–HCl, 1 mM EDTA (pH 7.6) to a final concentration of 100 μg/ml and the solution stored at −20°C.

2.4. *^{32}P-Labelled DNA*

Aliquots (10 μl) of DNA solution containing 1 μg of untreated or mAMSA-treated PY815 DNA or λ DNA were incubated with 8 units of calf intestinal alkaline phosphatase in 10 mM Tris–HCl buffer (pH 9.5) for 15 min at 25°C. Dephosphorylation was terminated by adding Na_2 EDTA to 1 mM and heating for 5 min at 100°C. The samples were then quickly chilled and a T4 polynucleotide kinase assay mixture (20 μl) was added giving final concentrations of 0.07 M Tris–HCl (pH 7.6), 0.01 M $MgCl_2$, 5 mM dithiothreitol and 2 μCi of [γ-^{32}P]ATP (spec. act. 5700 Ci/mmol). Phosphorylation was initiated with 1 unit of T4 polynucleotide kinase for 30 min at 37°C before the kinase was inactivated by heating at 100°C for 5 min.

2.5. *Separation of [5′-^{32}P]deoxyribonucleotides*

The ^{32}P-phosphorylated DNA was degraded to 5′-mononucleotides with pancreatic deoxyribonuclease and snake venom phosphodiesterase (each 100 μg/ml at 25°C for 1 h). Next, one A_{260} of each of the standard four non-radioactive 5′-deoxyribonucleotides was added to the sample before it was applied to a Whatman No. 3 mm chromatography paper and electrophoresed in 0.1 M formic acid/ammonium formate, 10 mM EDTA buffer (pH 3.5) at 1800 V (80 mA) for 1.5 h. The individual nucleotides were then quantitatively eluted and rechromatographed in a 1 M ammonium acetate/90% ethanol (30:70 v/v) solvent containing 0.01 M Na_2 EDTA and saturated with sodium tetraborate. Finally the UV-absorbing nucleotides were located and the radioactivity associated with each was measured in a Packard liquid scintillation spectrometer using toluene-based scintillant. The amounts of enzymes used in the above procedures were predetermined to give complete reactions in separate experiments.

3. RESULTS

To probe the availability of the 5′-hydroxyl residues of DNA fragments from mAMSA-treated PY815 cells, DNAs were prepared from untreated and mAMSA-treated cells. Aliquots of the resulting DNA preparations and of bacteriophage λ DNA were then dephosphorylated with calf intestinal phosphatase, the phosphatase inactivated and the DNAs rephosphorylated with ^{32}P using T4 polynucleotide kinase and high specific activity [γ-^{32}P]ATP. The resulting [5′-^{32}P]phosphorylated DNA was exhaustively hydrolysed with excess pancreatic deoxyribonuclease I and snake venom phosphodiesterase to degrade the DNAs completely to 5′-deoxyribonucleotides which were separated by high voltage paper electrophoresis. The individual nucleotides were recovered and subsequently chromatographed on paper using a borate-containing solvent system to separate 5′-ribonucleotides that contaminated the 5′-deoxyribonucleotides. Finally the radioactivity associated with the individual 5′-deoxyribonucleotides was measured. Paper chromatography with borate was necessary because DNA prepared and processed from cells contained polyriboadenylate sequences derived from messenger RNA which were not degraded and removed by pancreatic or T1 ribonuclease during the preparation of DNA. These sequences later became phosphorylated by [γ-^{32}P]ATP and produced [5′-^{32}P]riboadenylic acid which co-electrophoresed with 5′-deoxyadenylic acid after deoxyribonuclease and venom diesterase digestion. No other 5′-ribonucleotides were present.

When phage λDNA was processed in the above manner the major radioactive nucleotides produced corresponded to 5′-deoxyadenylic acid and 5′-deoxyguanylic acid, the known 5′-terminal nucleotides of phage λDNA (table 1a). Smaller amounts of radioactive deoxycytidylic acid and thymidylic acid were also produced either as a result of hydrolysis of λDNA by traces of deoxyribonuclease in the calf intestinal phosphatase or nicks in the original λDNA. The ^{32}P radioactivity associated with the 5′-(d)A and 5′-(d)G residues corresponded to 33% and 48% phosphorylation of the 5′-terminal (d)A and (d)G of phage λDNA, that is an average 40% reaction of the 5′-(d)A + (d)G, or a 20% reaction of true λDNA termini if random

Table 1

Phosphorylation of the 5′-terminal deoxyribonucleotides of phage λDNA and PY815 cell DNA by T4 polynucleotide kinase

DNA	Nucleotide	^{32}P incorporation (pmol ^{32}P/mg DNA)
(a) Phage λ	dA	11
	dG	16
	dC	6
	dT	7
(b) mAMSA-treated PY815 cells	dA	2.3
	dG	1.6
	dC	2.2
	dT	2.8
Untreated PY815 cells	dA	2.2
	dG	1.8
	dC	2.2
	dT	2.7

breakage had produced equal amounts of each of the four deoxyribonucleotides as additional spurious ends.

The results of a typical experiment phosphorylating DNA from untreated and mAMSA-treated cells are presented in table 1b. It can be seen that radioactivity was associated with all four deoxyribonucleotides, that there was no significant difference in the radioactivity associated with the individual nucleotides from untreated and treated cell DNA and that the four nucleotides were almost equally radioactive. From the mean size of DNA fragments produced from mAMSA-treated cells (approximately 2×10^8 daltons) [2] and assuming only a 20% efficient phosphorylation of DNA 5′-termini, equivalent to the lower figure for λDNA, it can be calculated that phosphorylation of mAMSA-treated cell DNA should have produced 0.4 mol [5′-^{32}P]nucleotide per 2×10^8 g DNA. The actual figures obtained corresponded to 1.82 mol [5′-^{32}P]nucleotide per 2×10^8 g DNA for both mAMSA-treated and untreated cell DNA, presumably because of random breakage of the DNAs during isolation or processing. Furthermore the treated and untreated cell DNAs and identical phosphorylated termini and there was no evidence of the expected extra 0.4 mol of [5′-^{32}P]nucleotide resulting from phosphorylation of additional breaks in DNA produced by mAMSA. Additional phosphorylation of this magnitude (approx. 25 000 cpm) should have been readily detected, especially if mAMSA showed any base specificity in cleaving DNA. Since the 5′-termini produced by random DNA breakage were phosphorylated it is clear that the phosphorylation of cellular DNA was successful. Therefore it is reasonable to conclude that mAMSA does not produce free phosphorylated or unphosphorylated (i.e., hydroxyl) 5′-termini when it induces breakage of cellular DNA, supporting the possibility that the termini are 'blocked', possibly by a topoisomerase that remains associated with the DNA [2].

Consistent with this view we have confirmed that the bulk of [^{3}H]thymidine-labelled DNA from mAMSA-treated cells is retained by the filters during the alkaline elution technique and that it is released following treatment with proteinase K as described by Zwelling et al. [2].

4. DISCUSSION

Studies of bacterial and eukaryote topoisomerases suggest that they become linked to 3′- or 5′-termini of DNA according to their origin and type and in some cases linkage to tyrosine–phosphate has been determined. Although the cleavage of DNA is non-random, no base sequence specificity has been detected. Rat liver and calf thymus topoisomerase I become linked to DNA 3′-termini. However, definitive data is not yet available for all of the enzymes in mammalian cells [5,6]. Without detailed knowledge of the nature of the linkage between the postulated topoisomerase and the terminus of cellular DNA in mammalian cells it is difficult to devise a procedure for selectively removing the terminal protein to label the DNA terminus and demonstrate its presence directly without degrading the associated DNA. In our experiments the use of proteinase K to prepare DNA from PY815 cells did not uncover 5′-termini in DNA from mAMSA-treated cells. Whether other proteases might do so remains to be determined. However most proteases would probably not hydrolyse a 5′-tyrosine–phosphodiester bond while usual chemical hydrolysis procedures lead to degradation of the DNA. Therefore we used an indi-

rect approach to the question of a possible topoisomerase linked to the 5′-termini of cellular DNA fragmented by mAMSA. The results support the view that the 5′-termini are 'covered' and unavailable for modification. To ascertain whether this is a result of topoisomerase attachment or some other modification will require more details of the specificity of the likely enzyme involved.

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